Protective Effect of pomegranate molasses (PM) Against Genotoxicity Induced by Benzoic acid (E-210) in human lymphocytes in vitro

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Abstract: The Middle Eastern diets contain many foods, among which the pomegranate molasses, are believed to have antioxidant effects, but yet, no research has been performed to evaluate the possible prophylactic role of this product to protect the genetic material of the cellular effects, therefore the current study was undertaken to investigate the prophylactic effects of pomegranate molasses in three concentrations (5, 10 and 15µg / mL) on human peripheral lymphocytes exposed to genotoxic effect of benzoic acid (E-210) with concentration of 500µg / mL, by using two types of cytogenetic studies, mitotic index (MI) and chromosomal aberration (CA) test through three types of transactions (before, after, and with treatment). Results showed that benzoic acid (E-210) induced chromosomal aberrations, and decreased mitotic activity in human peripheral lymphocytes. An interaction study of pomegranate molasses with benzoic acid (E-210), All the treatment led to reduce the toxic effect of benzoic acid (E-210). It is concluded that the pomegranate molasses, has antimutagenic potential that may prevent the mutagenic effect of various cytotoxic food additives.

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1. Introduction

Food additives are the substances that are added to food in order to prolong the shelf-life of the factory made foods by inhibiting the development of bacteria, fungi and other microorganisms.They are also used for some other purposes including coloring, flavoring, sweetening and thickening (Rekha & Dharman , 2011). There are about 2500 chemicals that function as food additives. However, the increased consumption of food additives may cause toxic reactions. It was reported that some food additives have genotoxic and carcinogenic effects in different tested organisms including bacteria, plants, human lymphocytes, mice and rats (Mamur *et al*., 2010, Yilmaz *et al* ., 2009, Mpountoukas *et al*., 2008).

Benzoic acid (E-210) is commonly used as an antimicrobial substance in many food products, added range between 150 and 1,000 mg / kg. like as fruit juice, syrup, pickle, ketchup, margarine, biscuit, waffle, cake and cream to preserve them from yeasts, mould and bacteria. Although epidemiological studies of food additives are important in the assessment of their toxicological risk to humans, they are difficult to be done because the exposure to a cannot be accurately assessed. Thus, risk assessment is largely depends on laboratory toxicity studies (Sasaki *et al* ., 2002).

A pomegranate (*Punica granatuml L.*) is a fruit-bearing deciduous shrub or small tree growing between five and eight meters tall (Nagaraju & Rao.,1990).The pomegranate *(Punica granatum L.)* belonging to pinaceae family which is widely distributed all over the world and has highly distinctive nutritional value.The pomegranate has extensively been used as a source of traditional remedies for thousands of years, and studies have shown that pomegranate has many potential effects including: bactericidal, antifungal, antiviral, immune modulation, vermifuge, stimulant, refrigerant, astringent, styptic, laxative, diuretic and anthelmintic effect. Moreover, it serves to decrease symptoms effects of cardiovascular diseases, diabetes, diarrhea, dysentery, asthma, bronchitis, cough, bleeding disorders, fever, inflammation, acquired immune deficiency syndrome, dyspepsia, ulcers, bruises, sores, mouth lesions, skin lesions, malaria, prostate cancer, atherosclerosis, hypertension, periodontal diseases, hyper lipidemia, denture stomatitis, male infertility, vaginitis, erectile dysfunction, alzheimer, obesity and infant brain ischemia (Lansky & Newman ., 2007, Reddy *et al*., 2007). Furthermore, pomegranate is an amazing source of cyaniding , delphinidin (both are anthocyanidins), caffeic acid, chlorogenic acid (both are phenolic acids), gallic acid, ellagic acid (tannic acids), luteolin, quercetin (flavones), kaempferol (a flavonol), naringenin (a flavanone) as well as 17-alphaestradiol, estrone, estriol, testosterone, betasistosterol, coumesterol, gamma-tocopherol, punicie acid, campesterol and stigmasterol in its juice (Vijayalakshmi *et al* ., 2012). In addition that peels and seeds oil chemopreventive and therapeutic potentials of this plant (Chalfoun-Mounayar *et al*., 2012; Sumathy *et al*.,2013). Therefore, the aim of this study is to determine the possible antimutagenic effect of pomegranate molasses against genetic toxicity resulting from benzoic acid in human blood lymphocytes, by using chromosomal aberration (CA) and mitotic index (MI) parameters.

2. Material and Methods

2.1 Chemicals

Benzoic acid CAS#:65-85-0, Chemical Formula: C6H5COOH. Benzoic acid was obtained from (Sigma, dissolved in 100 ml distilled water) to make the concentration of 500µg / mL (the amount used in foods) (Yilmaz *et al*., 2009).

2.2 Extraction Process of molasses.

We used fresh pomegranate juice (PJ) made from a pomegranate variety grown in Iraq. to production of pomegranate molasses, consisted of peeling the fruits, dispersing the grains and pressing them manually to have a juice. The juice is boiled for more than six hours in order to obtain a concentrated substance called "molasses.". (Gökçen *et al*., 1982), then attended concentrations (5, 10, and 15) µg /mL of pomegranate molasses.

2.3 Cell culture and treatments

Heparinized blood samples were collected from four healthy females, non-smokers, with age range 25 to 28). Whole blood for each samples (0.5 ml) was added to 5 mL of culture medium RPMI 1640 (Sigma, pH 6.8 to 7.0), supplemented with 10% fetal calf -serum, 10% antibiotic-antimycotic mixture and 1% phytohaemaglutinin of the final volume of cell culture (Carballo *et al*,1993). The culture tubes were then placed in the incubator at 37°C for 24 h. After 70 h of incubation, 0.1 mL of colcemid solution (1 µg/mL) was added to each tube and the contents were mixed by shaking the tubes gently, At the end of the incubation (72 h), the tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded. The pellet was resuspended using 10 mL of hypotonic solution (0.075 M KCl) and the tubes were incubated at 37°C for a further 4 min. The tubes were centrifuged again at 2000 rpm for 4 min and the supernatant was discarded. Following this, the pellet was resuspended using 10 mL of fresh fixative solution (methanol: acetic acid, 3:1). The tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded. This procedure was repeated three times. The pellet was resuspended and 0.5-1 mL of fresh, cold fixative solution was added to the tubes. Then 3 or 4 drops of cell suspension were dropped on cold wet glass slide. The slides were air dried and stained with 5% Giemsa.

2.4 Chromosomal Aberrations (CAs) Assay

The prepared slides were examined under the oil immersion lens of light microscope for 100 divided cells per blood lymphocytes culture, and the cells should be at the first metaphase stage of the mitotic division where the chromosomal aberrations are clear and the percentage of these aberrations was estimated.

2.5 Mitotic Index (MI) Assay

The slides were examined under high power (40 X) of compound light microscope and of divided and non-divided cells were counted and the mitotic index was calculated .

Mitotic index =no. of the dividing cells/ total no. of the cells (1000) ×100 (Ozkul *et al*.,2005).

2.6 Statistical Analysis

The data were expressed as mean ± SD. Statistical comparisons were performed by One-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test, and compared the differences between the moral test averages less significant difference (LSD) probability ($P < 0.05$) (SAS, 2010).

3. Results and Discussions

This experiment was designed to study the interaction of pomegranate molasses (PM) with the mutagenic effect of benzoic acid (E210) in human blood lymphocytes culture.

The results as shown in table (1) demonstrated that benzoic acid at 500 µg / mL, resulted in significantly decreased mitotic index (MI) in human lymphocytes, in table (2) benzoic acid induced a significant increase in the frequency of CAs in human lymphocytes compared with untreated control, benzoic acid induced five types of structural aberrations. The most common aberrations are chromatid breaks which indicates benzoic acid caused (DNA) double strand breaks and sister chromatid union which is the breakage followed by reunion of both sister chromatids at an identical site (Murli, 2003). There are many studies that showed the genotoxicity of different food additives in different cell lines (Mpountoukas *et al*., 2008; Yilmaz *et al*., 2008).

The mechanism operating in benzoic acid mediated mutation in human lymphocytes is currently unknown. However, genotoxicity may be mediated by inhibition of the activation of XRCC1, PARP-1 and DNA LIG3 proteins which are responsible for DNA repair or inhibition of OP18 stathmin activity that regulates microtubules (Yilmaz *et al*., 2012).

Table 1. The effect of interaction between pomegranate molasses (PM) and benzoic acid (E-210) on mitotic index on human blood lymphocyte culture (in vitro).

Test substance	Concentration µg /mL	Mitotic index
Control	0	**6.55 a**
Benzoic acid	500	**2.25 b**
Post – benzoic acid treatment	5	**4.55 c**
	10	**4.72 c**
	15	**5.35 d**
Pre- benzoic acid treatment	5	**5.55 d**
	10	**5.85 d**
	15	**6.34 a**
Simultaneous Treatment	5	**5.35 d**
	10	**5.54 d**
	15	**6.23 a**

Differences A, B, C, D, E are significant ($P < 0.05$) to compression rows

3.1 Interaction between pomegranate molasses (PM) and benzoic acid (E210) on human blood lymphocytes culture

An interaction study of pomegranate molasses with benzoic acid was carried out through three types of treatments (before, after and mixture) to determine the activity of pomegranate molasses extract in reducing the side effects of benzoic acid in vitro.

As shown in table (1, 2).The pre-treatment showed that the pomegranate molasses for all concentration used in this experiment (5,10, and 15) µg / ml has the ability to reduce the effect of the benzoic acid (E-210) in concentration 500 µg / ml in culturing human blood lymphocyte, post-treatment show different protection effect as shown in figure (1,2). Furthermore, treatment with a mixture of pomegranate molasses and benzoic acid (E-210) illustrate that the mixture was the ability to decrease the mutagenic activity of benzoic acid (E-210) Figure (1,2).

From these results it was found that the pomegranate molasses (PM) extract have the ability to reduce the effect of the benzoic acid (E-210) Figure (1,2). pomegranate molasses (PM) extract could be considered as bio antimutagen for its ability to decrease the effect of benzoic acid (E-210) in pre-treatment. bioantimutagen for its ability to decrease the effect

of benzoic acid in post-treatment. It was clear that post-treatment with pomegranate molasses extract may activate the suppressing agent or activate the promoters of DNA repair mechanism, or may increase the error free repair fidelity in the cell (Bronzetti *et al*., 1994).

Simultaneous treatment with mixture of pomegranate molasses (PM) extract and benzoic acid (E-210), Results showed that using the pomegranate molasses at the same time with benzoic acid can reduce the genotoxic effect. The ability to reduce chromosomal aberrations was similar to the reduction ability of pre-treatment Figure (1,2), which means that they have similar mechanism to reduce genotoxicity of benzoic acid (E-210). Many plant extracts were considered as desmutagen. It is possible to consider pomegranate molasses (PM) extract as a desmutagens for its ability to decrease the effect of benzoic acid (E-210) by may be due to the direct action of the compounds present in the extract of pomegranate molasses on benzoic acid by inactivating it enzymatically or chemically (Maurich *et al*., 2004). enzymatic inducers, mutagen scavenger or as antioxidant (Visioli *et al*., 2011). Polyphenols can interfere with the cellular detoxification systems, such as superoxide dismutases (SOD), catalase or besides, polyphenols can inhibit enzymes generating reactive oxygen species (ROS) as xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Jurenka , 2008). Flavonoids of pomegranate molasses (PM) extract have the ability to increase the detoxifying enzymes in the body and therefore reduce the effect of these mutagenic materials and their metabolites (Kanakis *et al*., 2005).Treatment with pomegranate molasses before the benzoic acid (E-210) and as a mixture provided protection ratios for MI and CAs more than ratios when given after benzoic acid (E-210). So, pomegranate molasses (PM) is classified as desmutagen in the first order, and bioantimutagen in the second order.

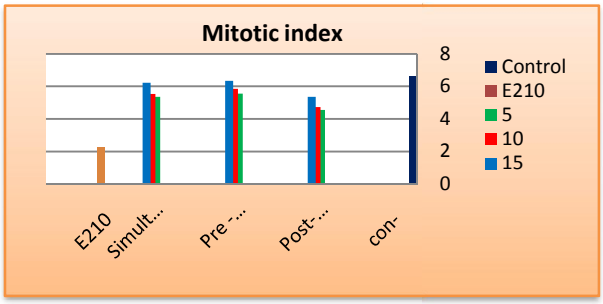

Fig (1): The protection ratios for mitotic index (MI) that provided by pomegranate molasses (PM) given before, after and as a mixture

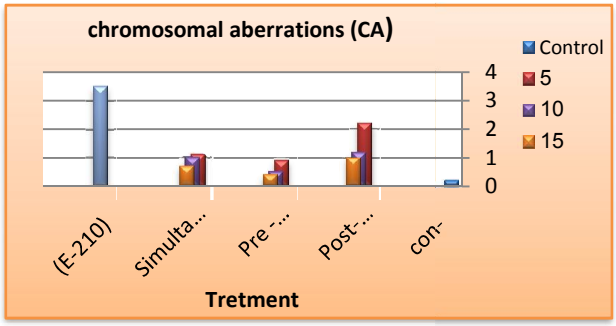

Fig (2): The protection ratios for chromosomal aberrations (CA) that provided by pomegranate molasses (PM) given before, after and

CONCLUSION

On the basis of our result we may conclude that all concentrations used in this experiment of pomegranate molasses, have shown significant protection against benzoic acid (E-210) - induced genotoxicity in human lymphocytes. It may be concluded that antimutagenic effects of pomegranate molasses may be due to it contains antioxidants such as alkaloids, tannins and flavonoids present in the product. Therefore, confirmed health benefits for pomegranate molasses to reduce mutagenicity caused by some food additives However, is needed further studies on active components and their effects on cell divisions for this product.

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Table (2): The effect of interaction between pomegranate molasses extract and benzoic acid (E210) on chromosomal aberrations (CA) in human blood lymphocyte culture (in vitro).

Test substance	Concentration µg /mL	Chromosomal Aberrations (CA)					Total %
		Dicenteric	chromatid Break	chromosome Break	Gap	Acentric	
Control	0	0	0	0	2	0	0.02 a
Benzoic acid	500	0.34	1.52	0.35	2.27	0.03	4.52 b
Post- benzoic acid treatment	5	0.14	0.85	0	0.98	0	1.97 c
	10	0	0.54	0	0.76	0	1.3c
	15	0	0.10	0	0.56	0	0.66 d
Pre - benzoic acid treatment	5	0	0.432	0	0.32	0	0.752 d
	10	0	0.05	0	0.09	0	0.14 d
	15	0	0.02	0	0.04	0	0.06 a
Simultaneous Treatment	5	0	0.64	0	0.55	0	1.19 c
	10	0	0.01	0	0.2	0	0.21 d
	15	0	0.05	0	0.01	0	0.06 a

Differences A, B, C, D, E are significant (P< 0.05) to compression rows

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